A METHOD FOR INTRODUCING SUBSTANCES INTO CELLS, AND USE OF SAID METHOD

Field of the invention

The present invention relates to in vitro and in vivo methods for introducing substances into a mammalian stem cell and/or progenitor cells, as well as to use of such methods.

Background of the invention

For several years it has been clear that cellular mechanisms exist that allow cells to internalize nucleic acids. A new approach for chemotherapy has been developed based on the fact that addition of defined oligonucleotides (antisense inhibitors) to cells in tissue culture has been shown to block specific gene expression.

Previous studies have established that short single stranded DNAs are rapidly internalized by a variety of cultured cells (Bennett, R. M., Gabor, G. T. and Merritt, M. M., J. Clin. Invest. 76, 2182-2190 (1985); Loke, S. L., Stein, C. A., Zhang, X. H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J. S. and Neckers, L. M., Proc. Natl. Acad. Sci. USA 86, 3474-3478 (1989); Yakubov, L. A., Deeva, E. A., Zarytova, V. F., Ivanova, E. M., Ryte, A. S., Yurchenko, L. V., and Vlassov, V. V., Poc. Natl. Acad. Sci. USA 86, 6454-6458 (1989); Iversen, P. L., Zhu, S., Meyer, A., and Zon, G., Antisense Res. Dev. 2, 211-222 (1992); Wu-Pong, S., Weiss, T. L., and Hunt, C. A. Pharmacol. Res. 9, 1010-1017 (1992); Chan, T. M., Framton, G and Cameron, J. S., Clin. Exp. Immunol. 91, 110-114 (1993)). There are reports of DNA receptor structures that mediate uptake and destruction of DNA in human leucocytes ((Bennett, R. M., Gabor, G. T. and Merritt, M. M. J. Clin. Invest. 76, 2182-2190 (1985)).

However, naked DNA, RNA and oligonucleotides are in general unable to cross cellular membranes in vivo (Bo-

ado, R. J., Tsukamoto, H. and Pardridge, W. M. J. Pharm. Sci. 87, 1308-1315 (1998). Therefore, several strategies based on the binding of DNA to soluble carriers, receptor structures or conjugates (e.g. DNA binding proteins, conjugates of poly-L-lysine and an integrin receptor ligand) mediating the interaction with a transmembraneous transport system (see e.g. WO 96/15811; WO 94/25608; Kato, Y. and Sugiyama, Y. Crit. Rev. Ther. Drug Carrier syst. 14, 287-331 (1997)).

In general, replacement of neurons following degeneration or damage is not a characteristic of the mammalian brain. Neuronal loss is thus considered permanent. Prolonged postnatal neurogenesis has been described in the granule cell layer of the hippocampal formation (Altman, J. and Das, G. D., J. Comp. Neurol. 124: 319-335 (1965); Altman, J. and Das, G. D., Nature 214: 1098-1101 (1967); Caviness, V. S. jr., J. Comp Neurol. 151: 113-120 (1973); Gueneau, G., Privat, A., Drouet, J., and Court, L., Dev. Neurosci. 5, 345-358(1982); Eckenhoff, M. F. and Rakic, P., J. Neurosci. 8: 2729-2747(1988)). Cell genesis and neurogenesis have recently been shown to persist well into adulthood in man (Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A., Nordborg, C., Peterson, D. A., Gage, F. H., Nature Med. 4:1313-1317 (1998).

Newborn neurons in the granule cell layer express markers of differentiated neurons and have morphological characteristics corresponding to differentiated granulae cells (Kaplan, M. S. and Bell, D. H., J. Neurosci. 4: 1429-1441 (1984); Cameron, H. A., Woolley, C. S., McEwen, B. S., and Gould, E., Neuroscience 56: 337-344 (1993); Cameron, H. A., Woolley, C. S., and Gould, E., Brain Res. 611: 342-346 (1993)). Furthermore, they establish axonal processes into the mossy fiber pathway and form synaptic connections with their targets in hippocampus CA3 (Seki, T. and Arai, Y., J. Neurosci. 13: 2351-2358 (1993); Stanfield, B. B., and Trice, J. E., Exp. Brain Res. 72: 399-406 (1988)). The hippocampus is associated with spatial

learning and memory (McNamara, R. K., and Skelton, R. W., Brain Res. Rev. 18: 33-49 (1993)). The proliferation of progenitor cells can be influenced by the administration of N-methyl-D-aspartate (NMDA) receptor antagonists or by the removal of the adrenal glands (Cameron, H. A., and Gould, E., Neuroscience 61: 203-209 (1994); Cameron, H. A., Tanapat, P., and Gould, E., Neuroscience 82: 349-354 (1998)). Plasticity is reduced with increasing age, and recent studies have demonstrated that proliferation of progenitor cells also is decreased but not completely abolished with age (Kuhn, H., Dickinson-Anson, H., and Gage, F. H., J. Neurosci. 16: 2027-2033 (1996)). Stem cells, isolated through a time consuming and laborious tissue culture procedure, from the adult rodent brain has recently been transplanted into the brain of adult animals where they differentiate into cells with neuronal characteristics (Suhonen, J. O., Peterson, D. A., Ray, J., and Gage, F. H., Nature 383:624-627 (1996)). There are so far no known stem cell markers that are usable for rapid isolation of stem cells or progenitor cells from the adult central nervous system. This fact inhibits the therapeutic use of stem cells in humans. So far the detection of stem cells rely on indirect detection method using modified nucleotides that incorporates in to the genome in dividing cells during the S-phase of the cell cycle. Thereafter, the phenotype of the progeny can be detected using immunohistochemical methods. The limitation with this way of identifying progenitor progeny is that these cells no longer possess the stem cell or progenitor cell properties meaning that these cells lack the ability to self renew and to give rise to neurons, astroglia, or oligodendrocytes. Alternative strategies to isolate stemcells from rodents, based on either unselective dye staining, immunosorting with antibodies against the protein nestin expressed by all cells surrounding the ventricles, or unselective infection with viruses carrying the gene for a selectable marker, was recently pub-

lished (Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., Frisen, J., Cell 96: 25-34 (1999)). Neither of these methods is highly efficient and thus unsuitable for rapid isolation of stemcells from small human tissue samples. Therefore, it is of importance to identify a usable marker or property allowing for rapid isolation of stem or progenitor cells for therapeutic purposes e.g. autologous neural transplantation.

Summary of the invention

Due to the fact that mammalian progenitor cells and stem cells from the adult CNS lack specific marker molecules it has up to now been virtually impossible to perform rapid detection and isolation of those cells. During the work leading to the present invention it was found that progenitor cells and stem cells from the adult brain possess a highly efficient mechanism for uptake of nucleic acids, such as DNA. It was also found that it is possible to use said transport system in order to mark or tag progenitor cells and stem cells via administration of e.g. double stranded DNA either in linear form or in circular form (plasmids) which is taken up by the progenitor cells or the stem cells via direct interaction between the DNA and the cells, without the use of facilitating drugs, carriers, soluble receptors or chemicals or any special devices. The DNA is not immediately degraded. Instead, if the plasmid DNA contains the necessary components for expression, the aforementioned cells can be detected by the expression of plasmid cDNAs. If the DNA/plasmid, containing suitable elements for expression, cDNA and promotor, is incubated in the presence of progenitor cells or stem cells, said DNA is taken up efficiently and the protein corresponding to the cDNA is expressed by the progenitor cells or stem cells.

The invention is based on the use of this nucleic acid transport system in progenitor cells and stem cells

for different purposes. According to the present invention, it is possible to transfer DNA without the help or aid of e.g. viral vectors. The invention provides new methods to isolate progenitor cells and stem cells in vivo and in vitro. This isolation may be based on the expression from plasmid containing cDNA of a protein that enables selective identification and isolation based on immunoreactivity, or on the expression by DNA of a protein that enables selective identification and isolation based on the expression of fluorescent proteins, including FACS sorting. The invention also provides new methods to transport different substances with e.g. pharmaceutical effects into progenitor cells and/or stem cells.

The object of the invention is thus a method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane in vitro whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell. Said method may be performed both in vitro and in vivo.

The method is particularly suitable for isolation of progenitor cells or stem cells from the adult brain, for gene therapy, for cell sorting and for diagnostic procedures.

The characterizing features of the invention will be evident from the following description and the appended claims.

There are several advantages with the present invention compared with known strategies based on the binding of DNA to a soluble carrier, a receptor structure or a conjugate (such as a DNA binding protein, a conjugate of poly-L-lysine and an integrin receptor ligand) mediating the interaction with a transmembraneous transport system. One important advantage is that the invention does not rely on the binding of DNA to any soluble receptors or

carriers. Another important advantage is that it allows for the selective labeling of cells, due to the fact that only cells with the above described inherent transport mechanism are transfected.

Detailed description of the invention

The transport mechanism upon which the present invention is based and which is found in mammalian stem cells and progenitor cells from the brain, including human stem cells and progenitor cells from the brain, can be utilized in order to transport single or double stranded DNA or RNA into a cell and subsequently allow for the DNA or RNA to remain intact and undegraded in the cell. Cells in which this transport mechanism is found are especially adult derived neural stem cells and progenitor cells.

By utilizing this transport it is thus possible to insert nucleic acids into said cells.

These nucleic acids may either be used for their ability to make it possible to identify and thus isolate progenitor cells and stem cells from other cells, or for their pharmaceutical effects.

As stated above, the present invention relates to a method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said substance is brought into contact with said cell, whereby it is taken up by the cell via the inherent transport mechanism of the cell. The method may be used both in vitro and in vivo. The cells used in the method according to the invention are preferably derived from an adult.

The substance to be introduced into a cell according to the method is or comprises e.g. a single or double stranded, linear or circular DNA, or a single or double stranded RNA. The substance may also be a fusion molecule comprising a nucleic acid part and a protein part, or an expression vector containing a specific cDNA. The expres-

sion "expression vector" used herein relates to all vectors or plasmids consisting of a double stranded DNA structure comprising cDNA for a specific peptide or protein. Once this expression vector is taken up by the stem cells or progenitor cells it will lead to the synthesis of said peptide or protein.

When the substance is an expression vector it is preferably, according to one embodiment of the invention further commented on below, that the cDNA gives rise to a peptide or protein that activate proliferation and/or differentiation and/or lineage determination of said cells.

As stated above, the method according to the invention may be performed both in vitro, e.g. in a tissue or cell culture, and in vivo. When the method is performed in vivo, the cells into which the substance is transported are preferably cells in the central nervous system.

The methods according to the invention may be used for several different purposes, both diagnostic and therapeutic.

When the method is performed in vitro, it is especially suitable for the identification of progenitor cells and stem cells. When the methods according to the invention are used for the purpose of identification it is preferable that the substance that is to be introduced into said cells gives rise to a detectable signal or to a peptide or protein that enables selective identification of stem cells and progenitor cells. Said peptide or protein may then in its turn give rise to a detectable signal, as the case is for e.g. a fluorescent protein, or a marker protein. Examples of suitable markers for stem cells or progenitor cells are protein components of the transport system, such as receptors and carriers. The detectable signal may also be obtained by the use of tagged substances, such as a radioactively tagged nucleic acid.

It is especially interesting to be able to identify, and thereafter isolate, progenitor cells and stem cells in samples constituted of e.g. different structures of brain tissue taken out of a patient or cells cultured from a patient.

Once the stem cells or progenitor cells have been identified, they can be isolated from the other types of cells in the sample by any appropriate method known to man skilled in the art. The isolated cells can then for example be used in different tests, for diagnostic purposes or be propagated and transplanted to a patient.

The in vivo method can be used in order to identify, and subsequently isolate, cells in vivo, in a way similar to the in vitro method described above. When the method is performed in vivo, it is possible to identify, and thus isolate, stem cells and progenitor cells in different structures of the intact brain

It is also possible to propagate stem cells and progenitor cells with the methods according to the invention. This propagation can be performed both in vitro and in vivo. The cells, which optionally first may have been identified and isolated with the methods according to the invention, are then brought in contact with a substance that comprises or gives rise to peptide or protein that, once it is taken up by the cells, activate proliferation and/or differentiation and/or lineage determination of said cells.

It is also possible to use both the in vitro and the in vivo methods according to the invention for gene therapy. The substance that is brought into contact with the cells, and subsequently is transported into the cells, may then be a pharmaceutically active substance. It may also give rise to a pharmaceutically active substance once it is taken up by the cells. The substance may then e.g. be an expression vector comprising cDNA encoding the pharmaceutically active substance. The pharmaceutically active substance produced by the cDNA once it is taken up

by the cell may be a peptide or protein that will get transported out of the progenitor cell or stem cell to affect surrounding tissue or cells. Examples of such a peptide or protein are trophic factors, or other proteins exerting a desired action on neighboring cells and tissues. The peptide or protein produced by the cDNA may also be a substance that will either activate or inactivate proliferation, differentiation or specific lineage determination of the progenitor cells or stem cells either in order to be able to more easily isolate progenitors or stem cells or in order to induce the genesis of new neurons, astrocytes or oligodendrocytes from progenitors or stem cells in the brain or within progenitors or stem cells in a tissue culture for concomitant use for transplantation of said cells to patients. It is also possible to use a substance constituted of a fusion molecule between a nucleic acid, that enables the transport into the cells, and a pharmaceutically active protein.

When the gene therapy is performed in vivo, it can be used for treatment of neurological insult, disease, deficit or condition in a patient. The term "treatment" used herein relates to both treatment in order to cure or alleviate a disease or a condition, and to treatment in order to prevent the development of a disease or a condition. The treatment may either be performed in an acute or in a chronic way. The term "patient", as it is used herein, relates to any human or non-human mammal in need of treatment according to the invention.

It is possible to produce medicinal products for treatment of conditions due to disturbances of the normal function of stem cells or progenitor cells by attaching a pharmaceutically active compound to a nucleic acid. The nucleic acid will, when it is brought into contact with a stem cell or progenitor cell, be taken up into the cell by the inherent transport mechanism of the cell, and since the pharmaceutically active compound is attached to the nucleic acid it too will be transported in to the

cell. Such medicinal products may also comprise other substances, such as an inert vehicle, or pharmaceutical acceptable adjuvants, carriers, preservatives etc., which are well known to persons skilled in the art. It is preferable that such medicinal products are administered to a patient by infusion into the cerebral ventricles through a surgically inserted canula or via a syringe inserted between lumbar vertebras and into the spinal fluid.

The methods according to the invention can also be used in order to test or screen a protein or a detectable signal. In a screening or test application the invention is used in with stemcells that take up DNA including cDNA coding for a protein of interest that are subject to screening or testing. Examples of proteins are receptors that can be used for screening new receptor agonists. The transport and uptake of and subsequent expression from plasmids in cells according to the invention can be used in detector devices and screening devices where expression of specific proteins like receptors or enzymes are desired. The advantage of the present invention compared with conventional transfection techniques in which drugs or compounds that facilitate DNA uptake are necessary for efficient uptake and expression, is the high efficiency and lack of need for drugs, compounds or chemicals to facilitate uptake and subsequent expression of proteins.

The invention will now be further explained in the following example. This example is only intended to illustrate the invention and should in no way be considered to limit the scope of the invention.

Brief description of the drawing

In the example below, reference will be made to the accompanying figure, wherein:

Figure 1A is a fluorescence photomicrograph showing the result of incubation of progenitor cells in medium with 50 µg/ml of a plasmid containing the cDNA for GFP;

Figure 1B a lightmicroscopic image showing the same result as figure 1A;

Figure 1C is a fluorescence photomicrograph showing the result of incubation of progenitor cells in medium with 50 µg/ml of another plasmid not containing the GFP gene; and

Figure 1D a lightmicroscopic image showing the same result as figure 1B.

Example

Expression by progenitor cells from mature rat brain of a fluorescent marker

The expression of green fluorescent protein (GFP) was examined in progenitor cells isolated from the adult hippocampus. Progenitor cells (Palmer, T. D., Ray, J. and Gage, F. H. (1995) Mol. Cell. Neurosci. 6: 474-486.) and cos-7 cells were cultured according to standard procedures and plated onto 1-inch circular coverslips coated with poly-D-ornithine and lamilin. The cells were incubated with plasmids containing the cDNA for GFP, and plasmids deficient of the GFP gene, respectively, in a humid atmosphere at 37°C with 5% CO_2 and 95% air for 10 minutes. The cells were cultured for 48 h, following DNA exposition.

Thereafter the expression of the fluorescent protein was detected using an inverted Leica DMIRB microscope equipped for fluorescence microscopy. The cells were viewed in the microscope using excitation of GFP at 488 nm using an Ar-ion laser (Spectra Physics model 2025-05, Sunnyvale, CA). The laser light was sent through a 488-line interference filter followed by a spinning disk to break the coherence and scatter the laser light. The laser was collected by a lens and sent through a fluorescein filter cube (Leica I-3) into the objective to excite the fluorophores. The resulting fluorescence was collected by the same objective and the image was detected by a 3-chip color CCD-camera (Panasonic) and recorded at

25 Hz frame collection rate by a Super VHS (Panasonic SVHS AG-5700). The CCD images were digitized from tape and processed for presentation.

When progenitor cells were incubated in medium with 50 µg/ml of a plasmid containing the cDNA for GFP for 10 minutes without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48h before detection, they were highly fluorescent. Figure 1A is a fluorescence photomicrograph showing this result, and Figure 1B shows the respective lightmicroscopic image.

In contrast, when progenitor cells from adult rat brain were incubated with other plasmids not containing the GFP gene, no fluorescence was observed. Figure 1C and 1D show the respective images when GFP deficient plasmid DNA were used.

Also, when progenitor cells were incubated with plasmid containing the gene expressing b-galactosidase 50 µg/ml, without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48 h before detection, cells expressed b-galactosidase activity.

It was also found that kidney-derived Cos-7 cells that were incubated in medium with 50 µg/ml of a plasmid containing the cDNA for GFP for 10 minutes without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48h before detection, lack expression of green fluorescent protein (GFP). Detection and experimental procedures for this experiment was identical to that for progenitor cells exposed to plasmid containing the cDNA for GFP.

Also, when cos-7 cells were incubated with plasmid containing the gene expressing b-galactosidase, without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48 h before detection, they displayed a lack of expression of b-galactosidase activity.

It is clear from the above experiments that progenitor cells from adult rat brain has a capacity to in vitro transport double-stranded DNA plasmids to their interiors, and to synthesize the proteins that the DNA sequence codes for.